Temporal and Lineage-specific Control of T Cell Receptor α/δ Gene Rearrangement by T Cell Receptor α and δ Enhancers

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Summary

To analyze the regulation of gene rearrangement at the T cell receptor (TCR) α/δ locus during T cell development, we generated transgenic mice carrying a human TCR δ gene minilocus. We previously showed that the presence of the TCR δ enhancer (E_δ) within the $J_\delta3$-C_δ intron was required to activate a specific step (V-D to J) of transgene rearrangement, and that rearrangement was activated equivalently in the precursors of $\alpha\beta$ and $\gamma\delta$ T cells. To further explore the role of transcriptional enhancers in establishing the developmental pattern of gene rearrangement at the TCR α/δ locus, we substituted the TCR α enhancer (E_α) in place of E_δ within the transgenic minilocus. We found that V-D-J rearrangement of the E_α^+ minilocus was restricted to the $\alpha\beta$ T cell subset. Further, we found that although V-D-J rearrangement of the E_δ^+ minilocus was initiated in the fetal thymus by day 14.5, V-D-J rearrangement of the E_α^+ minilocus did not occur until fetal day 16.5. Finally, whereas V-D-J rearrangement of the E_δ^+ minilocus is essentially completed within the triple negative population of postnatal thymocytes, V-D-J rearrangement of the E_α^+ minilocus is only initiated late within this population. Since the properties of minilocus rearrangement under the control of E_δ and E_α parallel the properties of V_δ-D_δ-J_δ and V_α-J_α rearrangement at the endogenous TCR α/δ locus, we conclude that these enhancers play an important role in orchestrating the developmental program of rearrangements at this locus.

Multipotential lymphocyte precursors differentiate into mature T cells via a series of intrathymic steps (1–4). V-(D)-J recombination of TCR gene segments is an essential component of this process. Two distinct types of T lymphocytes, bearing $\alpha\beta$ or $\gamma\delta$ TCR heterodimers, are generated. These subsets arise independently of each other within the thymus (5–7), and display distinct tissue distributions and appear to perform distinct functions in the periphery (8).

The four TCR genes are organized into three complex genetic loci, with TCR β and TCR γ gene segments unlinked, and TCR δ gene segments nested within TCR α gene segments at the TCR α/δ locus (9). V_δ-D_δ-J_δ and V_α-J_α rearrangements are therefore mutually exclusive on an individual chromosome. Typically, $\gamma\delta$ T cells display rearranged TCR γ, δ, and β genes but germline TCR α, whereas $\alpha\beta$ T cells display rearranged TCR α, β, and γ genes, with TCR δ deleted on both chromosomes. Thus, the pattern of gene rearrangement at the TCR α/δ locus is a distinguishing feature of $\alpha\beta$ and $\gamma\delta$ T cells. Furthermore, TCR gene rearrangements occur in an ordered fashion during thymocyte development. TCR δ, β, and γ rearrangements are initiated several days earlier than TCR α rearrangement in the fetal thymus (10–12), and are initiated in less mature phenotypic subsets of T cells than TCR α rearrangement in the postnatal thymus (13, 14).

The mechanisms by which the ordered and lineage-restricted rearrangement of antigen receptor genes are accomplished are of intense interest (15–17). As initially proposed by Blackwell et al. (18) and Yancopoulous and Alt (19), and as demonstrated in numerous studies over the last few years (20–27), transcriptional promoters and enhancers within TCR and Ig gene loci play important roles in controlling the accessibility of the recombinase machinery to chromosomal substrates. Thus, transcriptional promoters and enhancers are *cis*-acting elements that are likely candidates to impart developmentally unique regulation to V-D-J recombination at different TCR and Ig loci.

The precise mechanism by which these elements regulate accessibility to the recombinase is an open question. There is clearly a correlation between transcriptional and recombinational activity (18, 19, 28, 29). However, it is not clear whether enhancer-induced transcription is causal in promoting locus accessibility, or whether transcription is an independent consequence of enhancer-induced locus accessibility. Recent experiments suggest that rearrangement can in some instances occur in the absence of transcription (24, 26, 30–32),

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and that the core Ig μ enhancer can establish local accessibility in the absence of transcriptional activity (33). These data argue that enhancers can contribute multiple functions that may differentially affect transcription and rearrangement.

We are interested in determining the role of transcriptional enhancers and other *cis*-acting regulatory elements within the TCR α/δ locus in establishing the developmental pattern of V-(D)-J recombination at this locus. To study this issue, we previously generated transgenic mice carrying integrated versions of a human TCR δ gene minilocus (27). Analysis of V-D-J rearrangement of this minilocus revealed the following: First, the only rearrangement intermediate detected was V-D, arguing that the pathway of transgene rearrangement was V to D as a first step, and V-D to J as a second step. Second, the TCR δ enhancer (E_δ)[1] (34), located within the J-C intron, was essential for a specific step in transgene rearrangement, V-D to J. It is interesting to note that E_δ was unnecessary for V to D rearrangement. This was interpreted to indicate that the minilocus is divided into two discrete regulatory domains, and that E_δ controls access to the 3′ domain that contains J and C segments, but not to the 5′ domain that carries V and D segments. An insulator-like boundary between regulatory domains (35–37) was proposed to exist between D and J segments. Finally, V-D-J rearrangement, although T cell specific, was equivalent in $\alpha\beta$ and $\gamma\delta$ T cells. This argued that E_δ was activated to induce rearrangement in the precursors of both T cell subsets, and was not responsive to a lineage commitment signal that would restrict TCR δ gene rearrangement to $\gamma\delta$ T cells.

In the present study we further characterize the role of enhancer elements in orchestrating temporal and lineage-specific V-(D)-J rearrangement within the TCR α/δ locus. Specifically, we compared V-D-J rearrangement of the TCR δ gene minilocus under the control of either E_δ or the TCR α enhancer (E_α). We find that substitution of E_α for E_δ alters both the onset of rearrangement and the lineage specificity of rearrangement in ways that parallel the behavior of the endogenous TCR α/δ locus.

Materials and Methods

Transgenic Mice. The production of transgenic mice carrying an E_δ^+ minilocus was previously described (27). To construct the E_α^+ minilocus, a plasmid carrying the previously described enhancerless (E^-) minilocus was linearized by digestion with XbaI, treated with the Klenow fragment of *Escherichia coli* DNA polymerase I to generate blunt ends, and was treated with alkaline phosphatase. A plasmid containing a 1.4-kb KpnI-BamHI fragment of the human TCR α enhancer (38) cloned into the BamHI site of pSPCAT was kindly provided by J. Leiden (University of Chicago, Chicago, IL). This fragment was excised from the plasmid using BamHI digestion, was blunt ended by treatment with Klenow, and was cloned into the linearized E^- minilocus. In this position E_α precisely replaces E_δ (34) within the $J_\delta3$-C_δ intron (see Fig. 1). Minilocus DNA was purified as described previously, and was microinjected into fertilized C57BL/6 × SJL F2 eggs by the Duke University Comprehensive Cancer Center Shared Transgenic Mouse Facility. Progeny tail DNA was analyzed on slot blots (Schleicher & Schuell, Keene, NH) using a radiolabeled C_δ cDNA probe. Transgenes were maintained on a mixed C57BL/6 × SJL background.

Polymerase Chain Reaction. PCR reactions and Southern blot detection of PCR products was carried out using reaction conditions, primers, and probes described previously (27). The standard amount of template genomic DNA used in PCR reactions was 60 ng, with two exceptions. In the experiment presented in Fig. 3, 3 ng of template were used for the E_α^+ samples, and in the experiment presented in Fig. 6, 15 ng of template were used for all samples. Quantitative analysis of blots was accomplished using a Betascope (Betagen, Waltham, MA). For comparative quantification of rearrangement, V-D and V-D-J rearrangement signals were normalized to C signals for individual samples. Because the normalized ratios depend on V and C primer amplification efficiencies, as well as V and C probe specific activities and hybridization properties, the values reported are informative for comparisons between samples within an experiment, but are not informative for comparisons between samples in different experiments.

Antibodies, Flow Cytometry, and Cell Sorting. Monoclonal antibodies were used for purification of cell populations by a combination of cytotoxic elimination and cell sorting using a FACStar® (Becton-Dickinson and Co., Mountainview, CA) as described previously (27). The purity of sorted cell populations was in all instances assessed by immediate reanalysis of the sorted sample using a FACScan® (Becton-Dickinson and Co.). Contamination of cell populations was negligible.

Fetal Thymus Samples. Transgenic males were mated with 6–8-wk-old C57BL/6 × SJL F_1 females to produce timed pregnancies. The day of detection of the vaginal plug was designated as 0.5. Genomic DNA prepared from individual thymi was typed by PCR with C_δ primers to detect those carrying the transgene. Pooled positive samples were further analyzed for minilocus rearrangement.

Results

We previously generated transgenic mice carrying a human TCR δ minilocus (27). This minilocus, referred to here as E_δ^+, is 22.5 kb and contains germline $V_\delta1$, $V_\delta2$, $D_\delta3$, $J_\delta1$, $J_\delta3$, and C_δ gene coding segments, as well as E_δ (34) within the $J_\delta3$-C_δ intron. In this construct the $V_\delta1$ and $V_\delta2$ gene segments carry mutations that prevent a rearranged transgene from encoding a functional TCR protein and thereby influencing thymic development. For the present study, we generated a new version of the minilocus, referred to as E_α^+, in which a 1.4-kb KpnI-BamHI fragment carrying the human E_α (38) replaces the 1.4-kb XbaI fragment carrying E_δ (Fig. 1). We used this construct to generate transgenic mice, and obtained four founder mice that were bred to produce independent E_α^+ transgenic lines. Southern blot analysis showed that E_α^+ line J carries a single copy of the minilocus, lines L and M each carry two to three copies, and line N carries five to six copies (data not shown). Lines J, L, and M were used for the studies described below.

We analyzed rearrangement of these constructs by PCR using primers specific for the $V_\delta1$, $V_\delta2$, $J_\delta1$, and $J_\delta3$ gene segments (Fig. 1). As in our previous study (27), PCR using

[1] *Abbreviations used in this paper:* E^-, enhancerless; E_α, TCR α enhancer; E_δ, TCR δ enhancer; TN, triple negative.

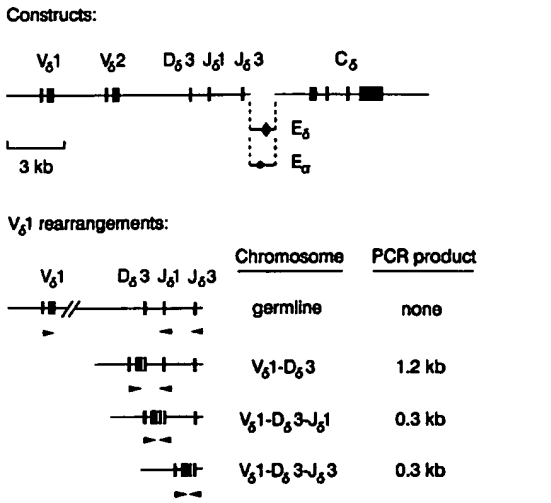

Figure 1. Schematic representations of germline and rearranged $E\delta^+$ and $E\alpha^+$ TCR δ minilocus constructs. Fragments of 1.4 kb carrying $E\delta$ (◆) or $E\alpha$ (●) were located within the $J\delta3$-$C\delta$ intron. The products generated from various $V\delta1$ rearrangements using $V\delta1$, $J\delta1$, and $J\delta3$ primers are depicted. A similar set of rearranged fragments are generated from $V\delta2$ rearrangements using $V\delta2$, $J\delta1$, and $J\delta3$ primers.

V and J primers allows detection of V-D-J rearrangements as 0.3-kb products that can be visualized by Southern blotting using $V\delta1$- and $V\delta2$-specific probes. PCR using either V primer with the $J\delta1$ primer allows detection of V-D rearrangements as 1.2-kb products that include the germline segment between $D\delta3$ and $J\delta1$. PCR with a pair of $C\delta$ primers and analysis with a $C\delta$ specific probe serves as an internal control for PCR efficiency. We have previously established that our PCR conditions allow quantitative comparisons of rearrangement levels in different samples (27).

Tissue Specificity of $E\delta^+$ Minilocus Rearrangement. We isolated genomic DNA from several lymphoid and nonlymphoid tissues of $E\alpha^+$ line L mice to assess the tissue specificity of V-D and V-D-J rearrangement (Fig. 2, *left*). Rearrangement was detected at highest levels in thymus, at lower levels in the spleen, and at barely detectable levels in the liver. No rearrangement was detected in the kidney, lung, or brain. We conclude that rearrangement is lymphoid specific, and that the low level of rearrangement detected in the liver is probably due to blood contamination. Similar tissue specificity had previously been obtained for $E\delta^+$ mice (27). The level of thymic minilocus rearrangement in $E\alpha^+$ line L is substantial; accounting for copy number, the level of rearrangement is equal to or greater than that observed in $E\delta^+$ line A. Minilocus $V\delta1$-$D\delta3$-$J\delta1$ rearrangement was previously shown to occur on about 30% of chromosomes in line A thymus (27).

To determine whether $E\alpha^+$ minilocus rearrangement was T cell specific, we fractionated line L splenocytes into pure T and B cells by a combination of cytotoxic elimination and cell sorting (Fig. 2, *right*). V-D-J rearrangement was found to be T cell specific, since it was detected at high levels in splenic T cells, but was not detected in splenic B cells. V-D rearrangement was enriched in splenic T cells, but was detected at low levels in splenic B cells. This behavior is identical to that of the $E\delta^+$ minilocus (27). The lack of absolute T cell specificity for TCR δ minilocus V-D rearrangement appears to parallel the lack of absolute B cell specificity for Ig heavy chain D-J rearrangement, which is the first step in V-D-J rearrangement at that locus (15–17).

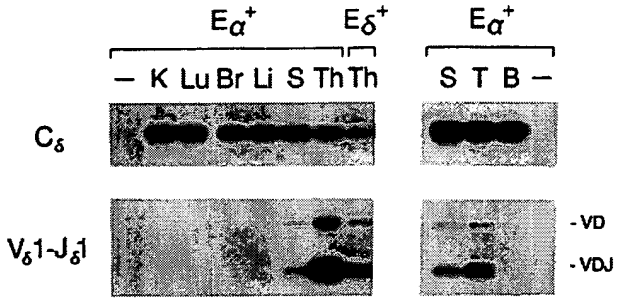

Figure 2. Tissue specificity of $E\alpha^+$ minilocus rearrangement. $V\delta1$ and $J\delta1$ primers were used to detect minilocus rearrangement by PCR, using genomic DNA from kidney (*K*), lung (*Lu*), brain (*Br*), liver (*Li*), spleen (*S*), and thymus (*Th*) from mouse L-7 (16-d-old), spleen(s), purified splenic T cells (*T*), and purified splenic B cells (*B*) of mouse L-11 (16-d-old), or no DNA (–) as templates. Rearrangement is compared to that in the thymus of $E\delta^+$ mouse A-48 (6-wk old). PCR using a pair of $C\delta$ primers served as an internal control. Southern blots of PCR products were probed with radiolabeled $V\delta1$ and $C\delta$ cDNA fragments.

Lineage Specificity of $E\delta^+$ Minilocus Rearrangement. Previous analysis of sorted $E\delta^+$ line B thymocytes demonstrated that V-D and V-D-J rearrangements occurred at equivalent levels in the $\alpha\beta$ and $\gamma\delta$ T cell subsets (27). We wanted to determine whether substitution of $E\alpha$ within the minilocus would confer $\alpha\beta$ subset specificity to minilocus rearrangement. Highly pure $\alpha\beta$ and $\gamma\delta$ T cell fractions were obtained by sorting of neonatal thymocytes of $E\alpha^+$ lines L and M. These were compared to similarly isolated $\alpha\beta$ and $\gamma\delta$ T cell fractions from two neonatal thymocyte preparations of $E\delta^+$ line A (Fig. 3). PCR analysis of minilocus rearrangement

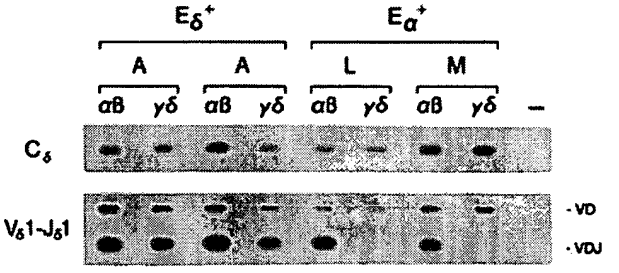

Figure 3. $E\delta^+$ and $E\alpha^+$ minilocus rearrangement in $\alpha\beta$ and $\gamma\delta$ thymocytes. Genomic DNA samples from sorted populations of $\alpha\beta$ and $\gamma\delta$ thymocytes, and a no DNA control (–), were amplified by PCR using the indicated primers. The two $E\delta^+$ samples analyzed represent pooled thymocytes from two 12-d-old line A littermates (*A, left*) and four 17-d-old line A littermates (*A, right*). The two $E\alpha^+$ samples analyzed represent pooled thymocytes from four 15-d-old line L littermates (*L*) and two 20-d-old line M littermates (*M*). Southern blots were probed with radiolabeled $V\delta1$ and $C\delta$ cDNA fragments.

in $E\delta^+$ mice revealed abundant V-D and V-D-J rearrangement in both $\alpha\beta$ and $\gamma\delta$ T cell samples. Levels of rearrangement, expressed as ratios of V-D-J/C and V-D/C, were very similar in all samples (Table 1). Analysis of $E\alpha^+$ mice revealed normal levels of V-D rearrangement in both $\alpha\beta$ and $\gamma\delta$ T cell samples. However, although V-D-J rearrangement was easily detected in the $\alpha\beta$ T cell samples, it was virtually undetectable in the $\gamma\delta$ T cell samples (Fig. 3 and Table 1). Similar results were also obtained in a third transgenic line carrying an $E\alpha^+$ minilocus (see Fig. 6). We previously found in mice carrying an E^- minilocus that although V-D rearrangement occurred at normal levels, V-D-J rearrangement was severely curtailed (27). Thus, in three different transgenic lines of mice, the $E\alpha^+$ minilocus behaves as if it were E^+ in $\alpha\beta$ cells but E^- in $\gamma\delta$ cells. $E\alpha$ therefore activates minilocus rearrangement selectively in developing $\alpha\beta$ T cells in these mice.

$E\delta$ and $E\alpha$ Determine the Timing of V-D-J Rearrangement during Ontogeny. Endogenous $V\delta$-$D\delta$-$J\delta$ and $V\alpha$-$J\alpha$ rearrangements are activated with distinct kinetics during fetal development (10–12). Since $E\delta$ and $E\alpha$ appear to play important roles in activating minilocus rearrangement, we sought to determine whether these elements could influence the time at which rearrangements are initiated during ontogeny. We therefore analyzed V-D and V-D-J rearrangement of the $E\delta^+$ and $E\alpha^+$ miniloci in series of fetal thymus samples obtained from timed pregnancies.

Analysis of $E\delta^+$ line A minilocus rearrangement using $V\delta1$ and $J\delta1$ primers revealed V-D rearrangement at the earliest timepoint examined, fetal day 13.5 (Fig. 4). At this time, V-D-J rearrangement is only barely detectable. However, there is a dramatic increase in V-D-J rearrangement starting at fetal day 14.5, and both V-D and V-D-J rearrangements further increase in abundance through day 17.5. Similar results were also obtained for line B $E\delta^+$ mice (data not shown). The observed early onset of rearrangement at the $E\delta^+$ minilocus closely parallels the onset of rearrangement at the endogenous murine TCR δ locus (12). Further, the elevated ratio of V-D/V-D-J rearrangement at early time points supports our previous inference that the predominant pathway of minilocus rearrangement consists of V to D as a first step,

Table 1. *Quantification of Minilocus V-D and V-D-J Rearrangement in $\alpha\beta$ and $\gamma\delta$ Thymocytes of $E\delta^+$ and $E\alpha^+$ mice*

	$E\delta^+$				$E\alpha^+$			
	A		A		L		M	
	$\alpha\beta$	$\gamma\delta$	$\alpha\beta$	$\gamma\delta$	$\alpha\beta$	$\gamma\delta$	$\alpha\beta$	$\gamma\delta$
V-D/C	1.4	1.0	0.72	0.81	0.69	0.38	0.63	0.46
V-D-J/C	3.3	2.2	3.0	2.5	3.5	0.00	1.5	0.02

Blot hybridization signals from the experiment shown in Fig. 3 were determined using a Betascope. Rearrangement was quantified as a ratio of V-D/C or V-D-J/C for each sample.

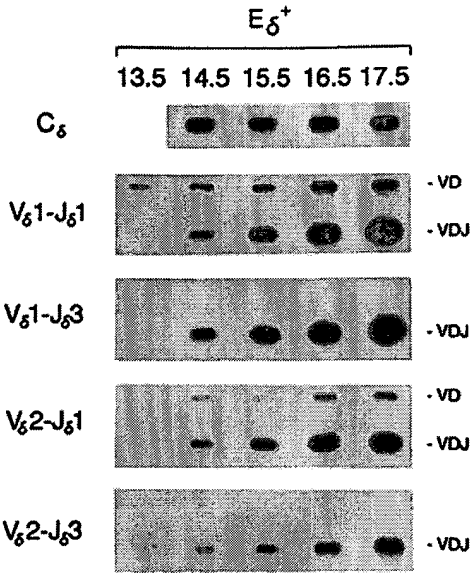

Figure 4. Time course of $E\delta^+$ minilocus rearrangement in the fetal thymus. Genomic DNA samples from line A fetal thymi isolated on days 13.5 through 17.5 of gestation were amplified by PCR using the indicated primers. Southern blots were probed with radiolabeled $V\delta1$, $V\delta2$, and $C\delta$ cDNA fragments. Due to differences in probe-specific activities and exposure times, the levels of $V\delta2$ and $V\delta1$ rearrangements cannot be directly compared. Note that a technical problem prevented analysis of the control $C\delta$ signal at fetal day 13.5.

and V-D to J as a second step. Consistent with this interpretation, minilocus D-J rearrangement could not be detected at any stage of fetal development by PCR with a 5′ $D\delta3$ and $J\delta1$ or $J\delta3$ oligonucleotides (data not shown).

Similar data for V-D and V-D-J rearrangement was obtained using the primer combinations $V\delta1$-$J\delta3$, $V\delta2$-$J\delta1$, and $V\delta2$-$J\delta3$. Very low levels of V-D-J rearrangement were detected with $V\delta2$ and $J\delta3$ primers as fetal day 13.5, but V-D-J rearrangements were readily detected with all primer combinations by fetal day 14.5. Thus, there was not a clear distinction between the onset of minilocus $V\delta1$ and $V\delta2$ rearrangement during murine fetal thymic development, despite the fact that $V\delta2$ is known to rearrange earlier than $V\delta1$ during human fetal thymic development (39). This apparent deregulation could occur because *cis*-acting elements that flank the V gene segments are by themselves insufficient to direct ordered rearrangement, or because they are not recognized appropriately in a murine background.

An analogous study was then performed on fetal samples from $E\alpha^+$ line J. Analysis of V-D and V-D-J rearrangements at fetal days 14.5 through 16.5 revealed that although V-D rearrangement is activated early and is therefore readily detected throughout this time period, activation of the V-D to J step is delayed until fetal day 16.5 (Fig. 5). Analysis of samples from $E\alpha^+$ line M yielded quite similar results. High levels of V-D rearrangement were detected from fetal day 14.5 onward, whereas V-D-J rearrangement, although first detected at very low levels on fetal day 15.5, was not detected at levels comparable to V-D rearrangement until fetal day 16.5 (data

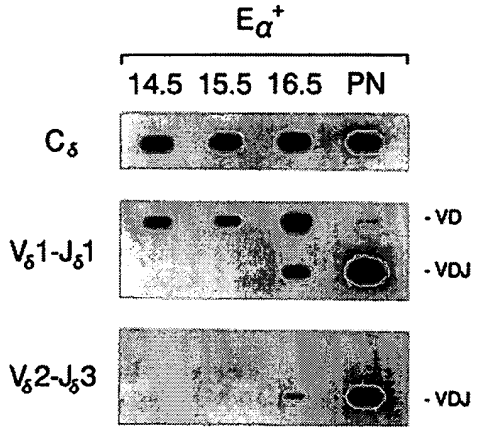

Figure 5. Time course of E_α^+ minilocus rearrangement in the fetal thymus. Genomic DNA samples from line J fetal thymi isolated on days 14.5 through 16.5 of gestation, and from 3 wk-old mouse J-70 postnatal thymocytes (*PN*), were amplified by PCR using the indicated primers. Southern blots were probed with radiolabeled $V_\delta1$, $V_\delta2$, and C_δ cDNA fragments. Due to differences in probe-specific activities and exposure times, the levels of $V_\delta2$ and $V_\delta1$ rearrangements cannot be directly compared.

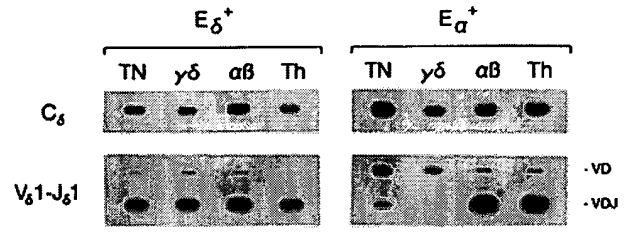

Figure 6. E_δ^+ and E_α^+ minilocus rearrangement in postnatal triple negative thymocytes. Genomic DNA samples from total thymocytes (*Th*) and sorted populations of triple negative (*TN*), $\alpha\beta$ and $\gamma\delta$ thymocytes were amplified by PCR using the indicated primers. Thymocytes from 11-d-old E_δ^+ mice A-449 and A-458 were pooled for sorting and analysis, as were thymocytes from 3-wk-old E_α^+ mice J-66 and J-70. Southern blots were probed with radiolabeled $V_\delta1$ and C_δ cDNA fragments.

not shown). Thus, V-D-J rearrangement of the E_α^+ minilocus is activated approximately 2 d later than was observed for the E_δ^+ minilocus. The delayed timecourse is consistent with the kinetics of V_α to J_α rearrangement at the endogenous TCR α locus (10, 11). These data indicate that E_δ and E_α are responsible for establishing the developmental time course of V-D-J rearrangement of the TCR δ minilocus, and from this we infer that these enhancers are important in establishing the temporal distinctions between the onset of TCR δ and TCR α gene segment rearrangement at the endogenous TCR α/δ locus.

E_δ and E_α Regulate the Onset of V-D-J Rearrangement in Populations of Postnatal Thymocytes. Triple negative (TN; $CD3^-CD4^-CD8^-$) thymocytes contain the precursors of both mature $\alpha\beta$ and mature $\gamma\delta$ T lymphocytes (40, 41). Whereas expression and rearrangement of the endogenous TCR δ locus is detected in relatively early subpopulations of TN thymocytes, expression and rearrangement of the endogenous TCR α locus is thought to occur in late TN thymocytes, as these cells begin to transit to the double positive ($CD4^+CD8^+$) stage (13, 14). To know whether E_δ and E_α confer stage specificity to minilocus rearrangement, we compared minilocus rearrangement in immature TN thymocytes to rearrangement in mature thymocyte subsets, including both $CD3^{hi}$ TCR $\alpha\beta^+$ and $CD3^{hi}$ TCR $\gamma\delta^+$ populations (Fig. 6). PCR analysis of subpopulations of E_δ^+ line A thymocytes using $V_\delta1$ and $J_\delta1$ primers revealed very similar levels of V-D and V-D-J rearrangement in both the immature and mature populations (Table 2). This suggests that E_δ^+ minilocus rearrangement is essentially completed within the TN population.

In contrast, analysis of the same populations in thymocytes of E_α^+ line J showed that V-D-J rearrangement in the immature TN cells is less than 10% of the level observed in the mature $CD3^{hi}$ TCR $\alpha\beta^+$ cells or in unfractionated thymocytes. This suggests that E_α^+ minilocus V-D-J rearrangement is initiated late within the TN population, and continues in more mature cells. We conclude that E_δ and E_α are responsible for differential activation of minilocus rearrangement within TN thymocytes, in a manner that parallels the rearrangement of the endogenous TCR δ and TCR α loci.

Discussion

To analyze the role of transcriptional enhancers in regulating TCR α/δ gene rearrangement during T cell development, we generated transgenic mice carrying a human TCR δ gene minilocus, and studied the rearrangement of this minilocus under the control of either E_δ or E_α. We found that the presence of E_δ or E_α altered V-D-J rearrangement of the reporter construct, as assessed by the lineage-specificity of rearrangement, the time course of rearrangement during fetal thymocyte development, and the phenotypic stage during which rearrangement occurs during postnatal thymocyte development. Specifically, whereas V-D-J rearrangement of the E_δ^+ minilocus is equivalent in $\alpha\beta$ and $\gamma\delta$ T cells, V-D-J rearrangement of the E_α^+ minilocus only occurs in $\alpha\beta$ T cells. Whereas V-D-J rearrangement of the E_δ^+ minilocus is initiated by fetal day 14.5, V-D-J rearrangement of the E_α^+ minilocus is not initiated until fetal day 16.5. Finally,

Table 2. *Quantification of Minilocus V-D and V-D-J Rearrangement in Triple Negative Thymocytes of E_δ^+ and E_α^+ mice*

	E_δ^+				E_α^+			
	TN	$\gamma\delta$	$\alpha\beta$	Th	TN	$\gamma\delta$	$\alpha\beta$	Th
V-D/C	0.27	0.32	0.17	0.14	0.68	0.68	0.30	0.22
V-D-J/C	2.4	3.3	2.6	2.4	0.30	0.10	3.9	3.3

Blot hybridization signals from the experiment shown in Fig. 6 were determined using a Betascope. Rearrangement was quantified as a ratio of V-D/C or V-D-J/C for each sample.
TN, triple negative.

whereas V-D-J rearrangement of the E_δ^+ minilocus is essentially completed within the TN population of postnatal thymocytes, V-D-J rearrangement of the E_α^+ minilocus is only initiated late within this population. We conclude that E_δ and E_α play important roles in orchestrating the complex developmental program of V-D-J rearrangement at the endogenous TCR α/δ locus.

We previously showed in mice transgenic for an E^- TCR δ minilocus that V to D rearrangement, but not V-D to J rearrangement, could occur independent of the presence of E_δ (27). Analysis of early fetal thymocytes and postnatal thymocytes of E_δ^+ and E_α^+ mice in the present study are consistent with this idea. V to D rearrangement is activated very early in both the E_δ^+ and the E_α^+ construct. Although this is very rapidly followed by E_δ-dependent V-D to J rearrangement in the E_δ^+ minilocus, E_α-dependent V-D to J rearrangement occurs fully 2 d later in the E_α^+ minilocus. Further, in the E_α^+ minilocus, V to D rearrangement, but not V-D to J rearrangement, occurs in the $\gamma\delta$ T cell subset. Our results argue that V to D rearrangement is activated in a very early population of thymocytes. One candidate could be the $Pgp1^+CD4^{low}CD8^-CD3^-$ population, which is the earliest precursor population in the thymus, and which contains cells with both T cell and B cell differentiation potential (42). This could account for the low level of minilocus V-D rearrangement that we routinely detect in B cell populations. Although this precursor population is known to carry germline TCR β and TCR γ genes, the configuration of the TCR δ gene has not been examined (43). We note that TCR δ V-D rearrangements are common in human precursor B-ALL cells (44–46).

We previously argued that the TCR δ minilocus studied here is composed of two independent regulatory domains, one containing V and D gene segments, and the other containing J and C gene segments (27). We further argued that E_δ selectively influenced J and C segment accessibility within the 3′ domain, and that the influence of E_δ was limited by the presence of an insulator between D and J segments. It was clear that V to D rearrangement could occur in the presence or absence of E_δ, and that the V-D to J step required E_δ. If E_δ only influenced the second step of the rearrangement process, then the total level of rearrangement (V-D) in E^- mice should be identical to the total level of rearrangement (V-D + V-D-J) in E^+ mice. However, because the various transgenes studied displayed a range of rearrangement levels that was influenced by the integration sites, it was impossible to evaluate whether the total level of construct rearrangement was influenced by the presence of E_δ. In this study, we could compare the properties of the E_α^+ minilocus in $\alpha\beta$ and $\gamma\delta$ cells carrying the same integrant. It is clear that V-D to J rearrangement (hence, J accessibility) occurs only in $\alpha\beta$ cells. However, it also seems apparent that the total level of construct rearrangement (V-D + V-D-J) is significantly higher in $\alpha\beta$ than in $\gamma\delta$ cells (see Figs. 3 and 5). This suggests the possibility that activation of E_α can influence the initiation of rearrangement events (i.e., V to D) in the 5′ domain of the minilocus. There are two ways in which this result can be rationalized with the two domain model of minilocus accessibility. First, it could be that E_α, because it is much more potent than E_δ, can overcome the influence of the putative insulator between D and J, and thereby influence accessibility in the 5′ domain. Second, it could be that under the control of E_α, the length of time that the minilocus is exposed to recombinase may be longer in developing $\alpha\beta$ cells than in developing $\gamma\delta$ cells. Our current data cannot distinguish between these possibilities.

In the human TCR δ gene minilocus studied here, human E_δ and E_α elements differentially activate construct V-D to J rearrangement. While this study was being completed, Capone et al. (47) reported a similar analysis of the role of murine E_β and murine E_α in V-D-J rearrangement of a hybrid murine TCR β/Ig μ locus. The regulation of that minilocus is different from ours, in that both D-J and V-D-J rearrangements are enhancer dependent. Despite the differences in minilocus organization and regulation, a comparison of the two studies indicates that human E_δ and murine E_β activate rearrangement at an early stage and in both the $\alpha\beta$ and $\gamma\delta$ T cell subsets, whereas human and murine E_α activate rearrangement at a later stage and in only the $\alpha\beta$ T cell subset. Analogous information is not yet available for TCR γ regulatory elements.

The observation that E_α directs V-D-J rearrangement of the TCR δ minilocus specifically within the $\alpha\beta$ T cell compartment could imply that E_α is activated by the binding of *trans*-acting factors specifically in the precursors of $\alpha\beta$ T cells. However, it is possible that lineage-specific activation of E_α need not be invoked to explain the behavior of the E_α^+ minilocus. Initial studies of E_α transcriptional activity in transfected cell lines indicated that a core murine E_α fragment did not display $\alpha\beta$ lineage specificity; rather, lineage specificity could be conferred by nearby *cis*-acting silencer elements (48, 49). Consistent with this, Capone et al. (47) found that the murine E_α fragment used in their study, which should not contain silencer elements, did not confer lineage-specific transcriptional activity to their construct. Nevertheless, this E_α fragment did confer lineage-specific rearrangement (47). The human E_α fragment used in our construct was originally reported to display $\alpha\beta$ lineage-specific transcriptional activity in transfection experiments (38). However, when we directly compared the ability of E_δ and E_α to activate transcription in the same reporter construct in both $\alpha\beta$ and $\gamma\delta$ T cell leukemias, we found that although E_α is more potent than E_δ, their relative activities did not vary dramatically between $\alpha\beta$ and $\gamma\delta$ cell lines (P. Lauzurica, and M.S. Krangel, unpublished observations). Finally, we note that none of the transcription factors that have been shown to interact with E_α are thought to display lineage-specific expression or activity (50).

Thus, core E_α fragments that appear to be transcriptionally active in both $\alpha\beta$ and $\gamma\delta$ T cells still confer $\alpha\beta$ specificity to V-D-J rearrangement. This could reflect a difference between the *cis*-acting enhancer elements required to activate transcription and those required to activate rearrangement (33). However, it is also possible that the ability of the core E_α fragment to confer $\alpha\beta$ specificity to minilocus V-D-J rearrangement could follow as a default from its temporal/stage

specificity, without requiring the involvement of any lineage-specific *trans*-acting factors that interact with the enhancer. Our experiments with the TCR δ minilocus, and numerous studies of the endogenous TCR α/δ locus, argue that TCR δ rearrangement occurs several days earlier than TCR α rearrangement in developing thymocytes (10–12, 14). TCR γ also initiates rearrangement at a stage prior to TCR α (10, 11). If the core E_α fragment is subject to delayed activation by a developmental clock in maturing thymocytes, and if functional TCR δ and TCR γ rearrangement can result in a shutoff of RAG (51, 52) gene expression, then E_α may become activated too late in developing γδ T cells to have any consequence for TCR gene rearrangement in these cells. The developmental clock could be understood, at least in part, on the basis of the expression characteristics of transcription factors thought to be important for core enhancer function. For example, c-Myb plays an important role in activation of E_δ (53) and is expressed in immature thymocyte subsets and early in ontogeny (54, 55), whereas Ets factors play an important role in activation of E_α (56), and are expressed in more mature thymocyte subsets and late in ontogeny (57).

Several experimental approaches have suggested that T cell lineage is determined before the onset of TCR gene rearrangement and that a lineage commitment step may in fact direct TCR gene rearrangement or expression (49, 58–60). However, it is not yet clear which events at TCR loci represent or respond to regulated lineage commitment steps. If the core E_α fragment activates rearrangement in a lineage-specific fashion by a proactive rather than default mechanism, activation of E_α could be an initial commitment step for the αβ pathway. This could be consistent with recent data arguing that αβ and γδ T cells can both arise from relatively late stage TN cells in the thymus (40). Alternatively, if the core E_α fragment simply responds to a developmental clock, then it cannot mediate an initial lineage commitment decision; rather, it can only function as a default. In this case, it would still be possible for TCR α rearrangement to be lineage determining if TCR α *cis*-acting elements other than the core E_α fragment (i.e., silencers [49]) are involved. The alternative possibility would be that activation of earlier events, such as TCR δ or TCR γ rearrangement and expression, would be lineage determining. Our data says that activation of TCR δ rearrangement per se is not regulated in a lineage specific fashion. However, TCR δ rearrangement could be prevented in a regulated manner by deletional rearrangement mediated by δ Rec and pseudo-J_α (61), if this event were activated early and in a directed fashion. Further, there is evidence that TCR γ transcription may be subject to lineage-specific control by silencer elements (60), suggesting this as a possible commitment step.

In summary, E_δ has the potential to activate TCR δ gene rearrangement, and E_β has the potential to activate TCR β gene rearrangement, in significant fractions of developing αβ and γδ thymocytes. This argues that control mediated at the level of E_β and E_δ cannot be involved in lineage determination. Regulated activation of δ Rec, TCR γ, and TCR α rearrangement and expression are all candidates that are not yet excluded as lineage determining events. The minilocus experiments show quite clearly that TCR α, β, and δ gene rearrangement events are at a minimum regulated by developmental clock-responsive enhancers. However, given the possibility that the core E_α fragment may mediate lineage-specific rearrangement by default, we note that none of the minilocus data formally excludes the possibility that lineage is determined in a stochastic way, as a consequence of functional TCR gene rearrangements, as initially proposed by Pardoll et al. (62).

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References

1. Nikiloc-Zugic, J. 1991. Phenotypic and functional stages in the intrathymic development of αβ T cells. *Immunol. Today.* 12:65.
2. Shortman, K. 1992. Cellular aspects of early T-cell development. *Curr. Opin. Immunol.* 4:140.
3. Rothenberg, E.V. 1992. The development of functionally responsive T cells. *Adv. Immunol.* 51:85.
4. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today.* 14:547.
5. Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson,

A.R. Clarke, M.L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor δ gene mutant mice: independent generation of αβ T cells and programmed rearrangements of γδ TCR genes. *Cell.* 72:337.
6. Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itohara, J.J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper, and S. Tonegawa. 1992. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature (Lond.).* 360:225.
7. Philpott, K.L., J.L. Viney, G. Kay, S. Rastan, E.M. Gardiner, S. Chae, A.C. Hayday, and M.J. Owen. 1992. Lymphoid development in mice congenitally lacking T cell receptor αβ-expressing cells. *Science (Wash. DC).* 256:1448.
8. Haas, W., P. Pereira, and S. Tonegawa. 1993. Gamma/delta cells. *Annu. Rev. Immunol.* 11:637.
9. Davis, M.M., and P.J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature (Lond.).* 334:395.
10. Raulet, D.H., R.D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature (Lond.).* 314:103.
11. Snodgrass, H.R., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature (Lond.).* 315:232.
12. Chien, Y.-H., M. Iwashima, D.A. Wettstein, K.B. Kaplan, J.F. Elliot, W. Born, and M.M. Davis. 1987. T-cell receptor δ gene rearrangements in early thymocytes. *Nature (Lond.).* 330:722.
13. Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay. 1989. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA.* 86:1614.
14. Held, W., C. Mueller, and H.R. MacDonald. 1990. Expression of T cell receptor genes in the thymus: localization of transcripts in situ and comparison of mature and immature subsets. *Eur. J. Immunol.* 20:2133.
15. Alt, F.W., E.M. Oltz, F. Young, J. Gorman, G. Taccioli, and J. Chen. 1992. VDJ recombination. *Immunol. Today.* 13:306.
16. Schatz, D.G., M.A. Oettinger, and M.S. Schlissel. 1992. V(D)J recombination: Molecular biology and regulation. *Annu. Rev. Immunol.* 10:359.
17. Lewis, S.M. 1994. The mechanism of V(D)J joining: lessons from molecular, immunological and comparative analyses. *Adv. Immunol.* In press.
18. Blackwell, T.K., M.W. Moore, G.D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F.W. Alt. 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. *Nature (Lond.).* 324:585.
19. Yancopoulos, G., and F. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. *Cell.* 40:271.
20. Ferrier, P., B. Krippl, T.K. Blackwell, A.J.W. Furley, H. Suh, A. Winoto, W.D. Cook, L. Hood, F. Costantini, and F.W. Alt. 1990. Separate elements control DJ and VDJ rearrangement in a transgenic recombination substrate. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:117.
21. Takeda, S., Y.-R. Zou, H. Bluethmann, D. Kitamura, U. Muller, and K. Rajewsky. 1993. Deletion of the immunoglobulin kappa chain intron enhancer abolishes kappa chain gene rearrangement in cis but not lambda chain gene rearrangement in trans. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2329.
22. Serwe, M., and F. Sablitzky. 1993. V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobulin heavy chain intron enhancer. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2321.
23. Oltz, E.M., F.W. Alt, W.-C. Lin, J. Chen, G. Taccioli, S. Desiderio, and G. Rathbun. 1993. A V(D)J recombinase-inducible B-cell line: role of transcriptional enhancer elements in directing V(D)J recombination. *Mol. Cell. Biol.* 13:6223.
24. Kallenbach, S., C. Babinet, S. Pournin, P. Cavelier, M. Goodhardt, and F. Rougeon. 1993. The intronic immunoglobulin κ gene enhancer acts independently on rearrangement and on transcription. *Eur. J. Immunol.* 23:1917.
25. Chen, J., F. Young, A. Bottaro, V. Stewart, R.K. Smith, and F.W. Alt. 1993. Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4635.
26. Lauster, R., C.-A. Reynaud, I.-L. Martensson, A. Peter, D. Bucchini, J. Jami, and J.-C. Weill. 1993. Promoter, enhancer and silencer elements regulate rearrangement of an immunoglobulin transgene. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4615.
27. Lauzurica, P., and M.S. Krangel. 1994. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor δ transgene. *J. Exp. Med.* 179:43.
28. Schlissel, M.S., and D. Baltimore. 1989. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell.* 58:1001.
29. Goldman, J.P., D.M. Spencer, and D.H. Raulet. 1993. Ordered rearrangement of variable region genes of the T cell receptor γ locus correlates with transcription of the unrearranged genes. *J. Exp. Med.* 177:729.
30. Hsieh, C.-L., R.P. McCloskey, and M.R. Lieber. 1992. V(D)J recombination on minichromosomes is not affected by transcription. *J. Biol. Chem.* 267:15613.
31. Hsieh, C.-L., and M.R. Lieber. 1992. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:315.
32. Engler, P., P. Roth, J.Y. Kim, and U. Storb. 1991. Factors affecting the rearrangement efficiency of an Ig test gene. *J. Immunol.* 146:2826.
33. Jenuwein, T., W.C. Forrester, R.-G. Qiu, and R. Grosschedl. 1993. The immunoglobulin μ enhancer core establishes local factor access in nuclear chromatin independent of transcriptional stimulation. *Genes Dev.* 7:2016.
34. Redondo, J.M., S. Hata, C. Brocklehurst, and M.S. Krangel. 1990. A T cell specific transcriptional enhancer within the human T cell receptor δ locus. *Science (Wash. DC).* 247:1225.
35. Kellum, R., and P. Schedl. 1991. A position-effect assay for boundaries of higher order chromosomal domains. *Cell.* 64:941.
36. Kellum, R., and P. Schedl. 1992. A group of scs elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* 12:2424.
37. Chung, J.H., M. Whitely, and G. Felsenfeld. 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. *Cell.* 74:505.
38. Ho, I.-C., L.-H. Yang, G. Morle, and J.M. Leiden. 1989. A T-cell-specific transcriptional enhancer element 3′ of C_α in the human T-cell receptor α locus. *Proc. Natl. Acad. Sci. USA.* 86:6714.
39. Krangel, M.S., H. Yssel, C. Brocklehurst, and H. Spits. 1990. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J. Exp. Med.* 172:847.
40. Petrie, H.T., R. Scollay, and K. Shortman. 1992. Commitment to the T cell receptor-αβ or -γδ lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur. J. Immunol.* 22:2185.

41. Godfrey, D.I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8-triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol.* 150:4244.
42. Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J. Exp. Med.* 174:1617.
43. Wu, L., R. Scollay, M. Egerton, M. Pearse, G.J. Spangrude, and K. Shortman. 1991. CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature (Lond.).* 349:71.
44. Griesinger, F., J.M. Greenberg, and J.H. Kersey. 1989. T cell receptor gamma and delta rearrangements in hematologic malignancies. Relationship to lymphoid differentiation. *J. Clin. Invest.* 84:506.
45. Biondi, A., P.F. di Celle, V. Rossi, G. Casorati, G. Matullo, G. Giudici, R. Foa, and N. Migone. 1990. High prevalence of T-cell receptor Vδ2-(D)-Dδ3 or Dδ1/2-Dδ3 rearrangements in B-precursor acute lymphoblastic leukemias. *Blood.* 75:1834.
46. Yokota, S., T.E. Hansen-Hagge, and C.R. Bartram. 1991. T-cell receptor δ gene recombination in common acute lymphoblastic leukemia: preferential usage of Vδ2 and frequent involvement of the Jα cluster. *Blood.* 77:141.
47. Capone, M., F. Watrin, C. Fernex, B. Horvat, B. Krippl, L. Wu, R. Scollay, and P. Ferrier. 1993. TCRβ and TCRα gene enhancers confer tissue- and stage-specificity on V(D)J recombination events. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4335.
48. Winoto, A., and D. Baltimore. 1989. A novel, inducible and T cell-specific enhancer located at the 3′ end of the T cell receptor α locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:729.
49. Winoto, A., and D. Baltimore. 1989. αβ lineage-specific expression of the α T cell receptor gene by nearby silencers. *Cell.* 59:649.
50. Leiden, J.M. 1993. Transcriptional regulation of T cell receptor genes. *Annu. Rev. Immunol.* 11:539.
51. Schatz, D.G., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell.* 59:1035.
52. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (Wash. DC).* 248:1517.
53. Hernandez-Munain, C., and M.S. Krangel. 1994. Regulation of the T-cell receptor δ enhancer by functional cooperation between c-Myb and core-binding factors. *Mol. Cell Biol.* 14:473.
54. Sheiness, D., and M. Gardinier. 1984. Expression of a proto-oncogene (proto-myb) in hematopoietic tissues of mice. *Mol. Cell Biol.* 4:1206.
55. Mucenski, M.L., K. McLain, A.B. Kier, S.H. Swerdlow, C.M. Schreiner, T.A. Miller, D.W. Pietryga, W.J. Scott, and S.S. Potter. 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell.* 65:677.
56. Ho, I.-C., N.K. Bhat, L.R. Gottschalk, T. Lindsten, C.B. Thompson, T.S. Papas, and J.M. Leiden. 1990. Sequence-specific binding of human Ets-1 to the T cell receptor α gene enhancer. *Science (Wash. DC).* 250:814.
57. Bhat, N.K., K.L. Komschlies, S. Fujiwara, R.J. Fisher, B.J. Mathieson, T.A. Gregorio, H.A. Young, J.W. Kasik, K. Ozato, and T.S. Papas. 1989. Expression of ets genes in mouse thymocyte subsets and T cells. *J. Immunol.* 142:672.
58. Winoto, A., and D. Baltimore. 1989. Separate lineages of T cells expressing the αβ and γδ receptors. *Nature (Lond.).* 338:430.
59. Dent, A.L., L.A. Matis, F. Hooshmand, S.M. Widacki, J.A. Bluestone, and S.M. Hedrick. 1990. Self-reactive γδ T cells are eliminated in the thymus. *Nature (Lond.).* 343:714.
60. Ishida, I., S. Verbeek, M. Bonneville, S. Itohara, A. Berns, and S. Tonegawa. 1990. T-cell receptor γδ and γ transgenic mice suggest a role of a γ gene silencer in the generation of αβ T cells. *Proc. Natl. Acad. Sci. USA.* 87:3067.
61. de Villartay, J.-P. R.D. Hockett, D. Coran, S.J. Korsmeyer, and D.I. Cohen. 1988. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. *Nature (Lond.).* 335:170.
62. Pardoll, D.M., B.J. Fowlkes, J.A. Bluestone, A. Kruisbeek, W.L. Maloy, J.E. Coligan, and R.H. Schwartz. 1987. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature (Lond.).* 326:79.